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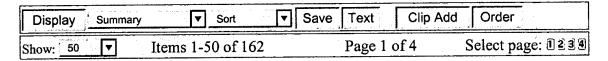
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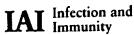
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Chelating agents inhibit activity and prevent expression of streptococcal glucan-binding lectins

, JS Singh, MY Galperin, D Drake, KG Taylor and RJ Doyle
Department of Microbiology and Immunology, University of Louisville, Kentucky 40292.

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Several of the cariogenic mutans streptococci produce cell wall- associated glucan-binding lectins (GBLs). The lectins bind alpha-1,6- linked glucans and have no affinity for other polysaccharides or anomeric linkages. When citrate or lactate was included in the growth medium, expression of the activities of the GBLs of Streptococcus cricetus and S. sobrinus was prevented. Furthermore, chelating agents, including citrate, lactate, EDTA, and acetylacetone, were able to reversibly inhibit glucan-induced aggregation of GBL+ streptococci. In addition, the chelating agents prevented sucrose-dependent streptococcal adhesion to glass surfaces and dispersed preformed adherent masses of the streptococci. Neither citrate nor other chelating agents modified the activities of glucosyltransferases. Expression of the lectin could only be achieved by the addition of manganous ion to the growth medium. Chloramphenicol and other metabolic inhibitors prevented synthesis of GBL in cells obtained from manganese- deficient medium and shifted to manganous ion-sufficient medium. The GBL may be a manganoprotein, the manganese of which may be perturbed, but not removed, by chelating agents. During synthesis of the GBL, manganous ion may be required in order for the protein to achieve an active conformation. Citrate or other chelating agents may have promise as anticaries agents.

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Inhibitory Effects of Plant Polyphenoloxidase on Colonization Factors of *Streptococcus sobrinus* 6715

M. M. Cowan, 1, E. A. Horst, S. Luengpailin, and R. J. Doyle²

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ABSTRACT

Exogenously added polyphenoloxidase (ÉC 1.14.18.1), an enzyme which oxidizes tyrosine residues and is commonly found in many dietary components, abolished the aggregation of *Streptococcus sobrinus* 6715 by high-molecular-weight dextran. The enzyme decreased glucan-binding lectin and/or glucosyltransferase I activities.

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TEXT

In the past three decades, rapid progress has been made in the understanding of microbial adhesion (8). A wealth of research has established that Streptococcus sobrinus must attach to glucans deposited on the tooth surface for successful colonization of the oral cavity (4, 6,

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17). For this purpose it uses a glucan-binding lectin (GBL) and a family of glucosyltransferases (GTFs). GTFs are composed of a C-terminal glucan-binding domain supplemented by an N-terminal catalytic peptide (7). Both GBL and GTFs have been shown to possess critical tyrosines in their glucan-binding sites (15). In this study we investigated the effect on adhesion of bacterial pretreatment with polyphenoloxidase (PPO) (EC 1.14.18.1). PPO is an enzyme found in many plant species, including most noncitrus European fruits and many vegetables (18). It possesses two activities, oxidizing a variety of phenolic substrates, including tyrosine, to L-dihydroxyphenylalanine and then to quinones. This action results in browning when it occurs in fruits, such as apples and bananas. Plants and invertebrates may use the PPO system, with its resultant tannin production, as a defense against invasion by predators, such as fungi and insects (11,

<u>12</u>).

S. sobrinus 6715 was maintained and grown either on tryptic soy agar or in the defined medium of Terleckyj et al. (16). The standard rate assay of Drake et al. (3) was used to study the interaction of S. sobrinus 6715 GBL with high-molecular-weight dextran. Briefly, bacterial suspensions were mixed with dextran T-2000 (10 µg/ml), and the decrease in optical density was continuously monitored spectrophotometrically for 5 min. Absorption at 540 nm was used to calculate ln(A/A₀) (A, observed optical density; A₀, optical density at time zero), which was plotted versus time in minutes. Each sample was assayed in triplicate. For enzyme treatments, cells were incubated with PPO (from mushrooms; Worthington Biochemical Corporation, Freehold, N.J.) (180 to 1,260 U/ml) for 1 h at 37°C.

Figure 1 depicts the decrease in absorption for control and PPO-treated (464 U/ml) S. sobrinus 6715 after mixing with glucan (T-2000). Bacteria in this experiment were grown in complex medium. Cells grown in defined medium required sevenfold-lower concentrations of PPO for inhibition (data not shown). PPO pretreatment reduced aggregate formation to approximately the level seen when a competitive binding inhibitor, low-molecular-weight glucan (dextran T-10), was included in the reaction (Fig. 1). When dextran T-10 was added to cells before enzyme treatment, the action of PPO was blocked. Addition of glycogen prior to PPO treatment had no effect on PPO's activity.

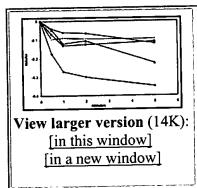


FIG. 1. Inhibition of aggregation of S. sobrinus by high-molecular-weight dextran after treatment with PPO. High-molecular-weight dextran (dextran T-2000) was added as a last step in all tubes. ♠, control (S. sobrinus); ×, low-molecular-weight dextran (T-10) plus S. sobrinus; ♠, S. sobrinus pretreated with PPO; ♠, dextran T-10 added to S. sobrinus before PPO treatment; ■, glycogen added to S. sobrinus before PPO treatment. Experiments were performed in triplicate.

The following known PPO inhibitors prevented the enzyme from abolishing aggregation of S. sobrinus by glucan: EDTA (5 mM), 100% decrease in PPO activity; potassium chloride (200 mM), 100% decrease; polyvinylpyrrolidone (500 μ g/ml), 100% decrease; ascorbic acid (3 mM), 100% decrease; and lactic acid (10% [wt/vol]), 91% decrease. Protease inhibitors, phenylmethylsulfonyl fluoride (500 μ M) and leupeptin (500 μ g/ml), were also tested to ensure that the activity was not due to possible contaminating proteases in the enzyme batches. Neither decreased the action of PPO. Incubation of inhibitors with S. sobrinus had no effect on control glucan-dependent aggregation. All inhibitors were from Sigma.

PPO was mixed (by gentle vortexing) with S. sobrinus-glucan complexes after 30 min of control aggregation (Fig. 2). Reformation of aggregates was significantly retarded with respect to the control bacteria. After another 30 min the complexes were vortexed again (to disrupt aggregates) with no further addition of PPO. PPO-containing tubes continued to show slower and less complete aggregation, suggesting either that PPO enzymatically altered the binding site or that it bound with a higher affinity than dextran.

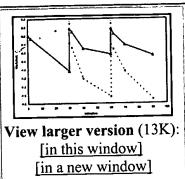


FIG. 2. Prevention of reaggregation of S. sobrinus by addition of PPO. , control, S. sobrinus plus glucan T-2000; , S. sobrinus plus glucan T-2000 plus PPO. Arrow 1 shows when PPO was added with vortexing, and arrow 2 shows when the complexes were vortexed with no further addition of PPO. Experiments were performed in triplicate.

Growth vessel pellicle formation, mediated by the combined activity of GTFs and GBL (17), was investigated. Bacteria were inoculated into 5-ml tubes of tryptic soy broth with and without sucrose (200 mM) and/or PPO (1.0 mg/ml). After 18 h of growth, tubes were emptied, stained with crystal violet, and visually examined. Only PPO-containing cultures produced no pellicles in the presence of sucrose (data not shown).

The ability of PPO to reduce the activity of partially purified GTFs was assayed as follows. PPO-treated (50 U/ml) and untreated crude GTFs obtained through ammonium sulfate precipitation of *S. sobrinus* culture supernatant were subjected to nondenaturing electrophoresis in polyacrylamide. The presence of glucan-binding bands was demonstrated by incubating duplicate gels in fluorescein isothiocyanate-conjugated glucan T-10 (2 mg/ml). GTF activity was assayed by incubating gels in sucrose followed by development with Schiff's reagent. Of four discrete bands which bound glucan, two (molecular weight, 145,000 and 135,000) demonstrated GTF activity. The higher-molecular-weight band, corresponding to the reported size (15) of GTF-I (an isoenzyme producing insoluble glucan), lost activity after incubation with PPO. Glucan-binding activity has been shown to reside both on glucan-binding proteins (with no catalytic activity) and on the C-terminal end of GTFs (5, 7, 15). Further study is needed to determine which of the family of glucan-binding proteins is affected by PPO. A combination of GBL and GTF-I inhibition could have a potentially powerful effect on oral ecology.

Disk diffusion (100 U of PPO) and broth dilution assays (in tryptic soy broth) of PPO (highest PPO concentration = 464 U/ml) showed that PPO did not prevent growth of *S. sobrinus* (data not shown).

PPO is a copper-requiring metalloenzyme. Therefore, metal-chelating agents have been found to be inhibitory to its activity (19). The PPO inhibition by EDTA, ascorbic acid, and lactic acid seen in this study supports this finding and suggests that naturally occurring chelators, such as lactic acid manufactured by oral streptococci, could have similar effects in vivo and could conceivably be an adaptive response by the bacteria to the constant presence of dietary PPO originating in fruits and vegetables. Various studies report that persons consuming large quantities of fruits and vegetables do indeed have lower caries rates (10). It is plausible, based on the present results, that PPO may play some role as an anticaries agent.

There are many examples of the importance of tyrosine in carbohydrate-binding sites of microbial proteins (1, 2, 9, 13, 14, 20, 21). If tyrosine is indeed a "consensus" residue necessary for the specific binding of multiple microbial pathogens to host tissues, alteration of tyrosine could represent a broad-spectrum approach to the prevention and interruption of microbial attachment and biofilm formation.

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FOOTNOTES

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